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Cancer with the Clot-binding Peptides CLT1 and CLT2

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14. ABSTRACT Hormone refractory prostate cancer is virtually incurable as it responds only poorly to conventional chemo- and radiation therapy. It is our goal to improve the overall outcome of prostate cancer through targeted delivery of drugs which are conjugated to homing peptides with high affinity for specific binding sites expressed in prostate tumors. We recently identified two peptides, CLT1 (CGLIIQKNEC) and CLT2 (CNAGESSKNC), that accumulate in tumor interstitial spaces where they appear to associate with proteins specific for plasma clotting. The scope of this grant was to analyze the interaction of CLT1 and 2 with the prostate tumor extracellular matrix. To this end, we performed an in vivo alanine scan to identify the amino acid sequence that mediates binding of CLT and CLT2 to the prostate tumor stroma. So far, our results suggest that AKN is part of the CLT1 tumor homing motif. Further studies are needed to confirm this result and to determine the CLT2 tumor binding function.				
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Introduction

Hormone refractory prostate cancer is virtually incurable as it responds only poorly to conventional chemo- and radiation therapy (1). These treatments are usually underdosed because they are not only toxic for tumor cells but also for healthy proliferating tissues. It is our goal to improve the overall outcome of prostate cancer through targeted delivery of drugs which are conjugated to homing peptides with high affinity for specific binding sites expressed in prostate tumors. The tumor stroma is an excellent target for tumor-homing peptides because it has a different composition than normal connective tissue (2). One such specific component is clotted plasma which regularly infiltrates the interstitial spaces of tumors including prostate cancer (3). We recently identified peptides that specifically bind to clot in the tumor stroma by screening a phage library on clotted plasma *in vitro* (4). The clot-binding peptides CLT1 (CGLIIQKNEC) and CLT2 (CNAGESSKNC) accumulate in tumor interstitial spaces where they appear to associate with proteins specific for plasma clotting such as fibrin and plasma fibronectin. CLT1 and 2 display structural similarities in the C-terminal half of their molecules (CLT1: CGLII**QKNEC**; CLT2: CNAGE**ESSKNC**), which led us to hypothesize that Q/SKN is the shared tumor homing motif of CLT1 and 2. The scope of this grant was to analyze the interaction of CLT1 and 2 with the prostate tumor extracellular matrix. To this end, we performed an *in vivo* alanine scan to identify the amino acid sequence that mediates binding of CLT1 and CLT2 to the prostate tumor stroma.

Body

Goal: Determine if XKN is the tumor-binding sequence homology shared by CLT1 and CLT2.

Tasks (as described in Statement of Work):

1. Synthesize CLT1 and 2 variants with alanine substitution of each residue.
2. Grow tumor xenografts including injection of alanine-substituted CLT peptides.
3. Prepare tumor and organ tissue sections for analysis by fluorescence microscopy.
4. Repeat *in vivo* tumor-binding study with CLT peptide variants that display increased or decreased tumor-homing properties including tissue processing and analysis by fluorescence microscopy.

Task 1: All peptides were purchased from Primm Biotech, Cambridge, MA. CLT1 and 2 are cyclic decapeptides with the sequence CGLIIQKNEC (CLT1) and CNAGESSKNC (CLT2), respectively. Cyclization of the peptides is induced by cysteines at the C- and N-terminus, which form intramolecular disulfide bonds in the presence of oxygen. Linear versions of CLT1 and 2 (named ACLT1/2) were generated by replacing the C- and N-terminal cysteine with alanine. As a starting point for the alanine scan, we focused on the QKN motif of CLT1

(structure: CGLIIQKNEC) and the homologues SKN motif in CLT2 (CNAGESSKNC). The following fluorescein-conjugated peptides were commissioned: CGLIIAKNEC (CLT1 QA), CGLIIQANEC (CLT1 KA), CGLIIQKAEC (CLT1 NA), CNAGESAKNC (CLT2 SA), CNAGESSANC (CLT2 KA), CNAGESSKAC (CLT2 NA). All peptides were conjugated to carboxyfluorescein (CF) via a 2-aminoethoxy-2-ethoxyacetic acid (AEEA)-linker for detection of tumor binding by fluorescence microscopy. Peptide quality was monitored by mass spec (single peak at ca. 1500 dalton) and HPLC (single peak at ca. 18 minutes).

Task 2: We initially planned to use PPC1 xenografts for the *in vivo* alanine scan but found during the course of our studies that both CLT1 and CLT2 bound more strongly to DU145 prostate tumor xenografts (Figure 1). Consequently, DU145 xenografts were generated by injecting 2×10^6 tumor cells into the flank of athymic nude mice. This resulted in a tumor take rate of ca. 80 %. Alanine substituted CLT1 and CLT2 variants were injected into the tail vein when the tumors reached a diameter of 0.5-1 cm. CLT1 and 2 were used as positive controls, linear ACLT1 and 2 as negative controls. Each peptide was injected into 2 mice at 500 μ g. Four hours after peptide injection, mice were anaesthetized and perfused through the heart. Tumors were isolated, fixed in paraformaldehyde over night and frozen in OCT.

Task 3: We analyzed histological sections from paraformaldehyde-fixed DU145 prostate tumor tissues by fluorescence microscopy. Tumor binding was determined based on the specific fluorescence produced by the fluorescein-tagged peptides in the tumor stroma. We assessed tumor binding of the alanine-substituted CLT1 peptides in relation to "wildtype" CLT1 as a positive control and ACLT1 as a negative control. Based on these criteria, we found that tumor homing was increased with CLT1 QA and decreased with CLT1 NA or CLT1 KA (Figure 2). CLT2 SA seemed to bind more strongly to the tumor stroma than "wildtype" CLT2, whereas CLT2 NA showed no obvious difference in homing compared to wildtype CLT2 (Figure 3). The tumor homing function of CLT2 KA was inconclusive. ACLT2, which was tested for the first time, was completely negative as expected. At this stage, our results suggest that AKN is an important part of the CLT1 tumor homing motif, but AKN is not critical for CLT2 tumor homing.

Task 4: We have not been able to complete this task within 1 year due to initial problems with the PPC1 tumor model and the ambitious nature of the project.

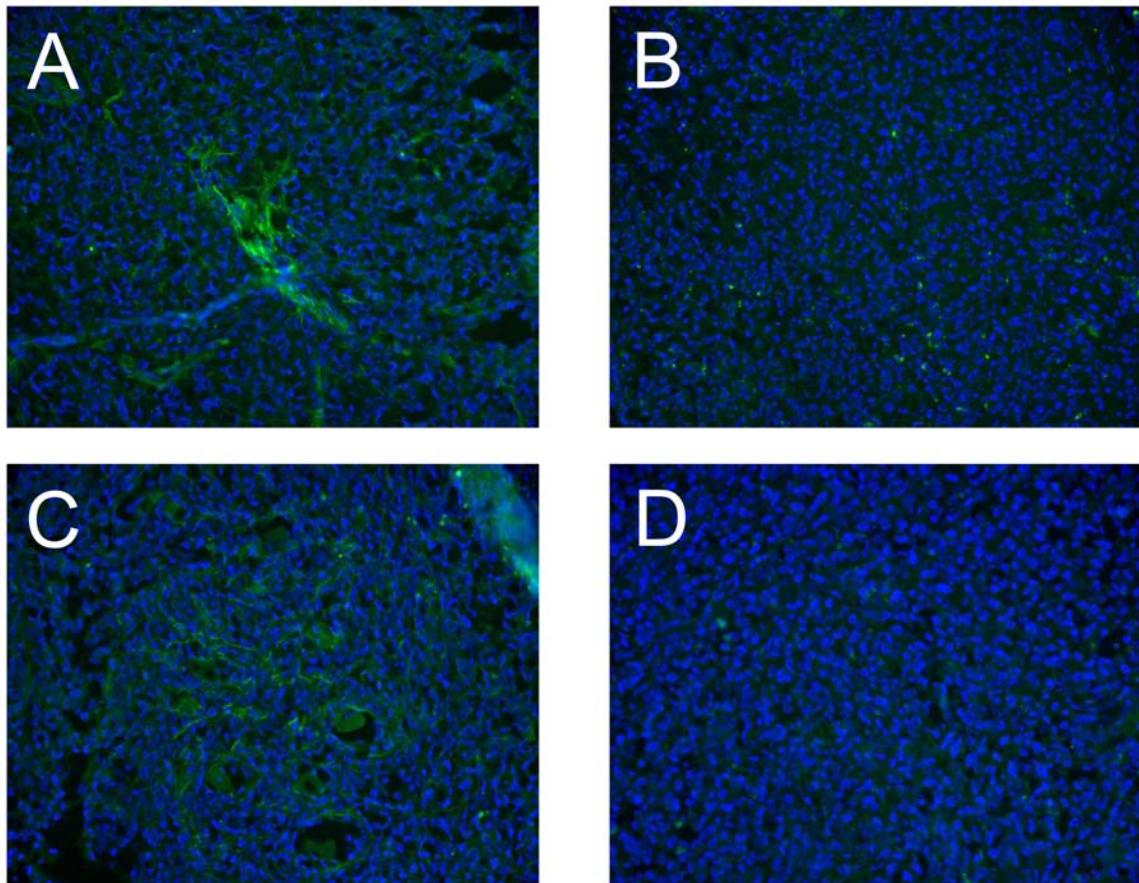


Figure 1 Mice carrying DU145 (A-B) or PPC1 (C-D) prostate tumor xenografts were tail vein-injected with 500 μ g CF-conjugated CLT1 (A, C) or ACLT1 (B, D). After 4 h, the mice were perfused with PBS and tumors were isolated. Histological analysis using fluorescence microscopy showed that CLT1 (green) distributed in a network pattern within the stroma of DU145 (A) and, to a lesser extent, of PPC1 (C) prostate tumor xenografts. (B, D) The fluorescein-conjugated control peptide ACLT1 did not bind to the tumor tissue. Nuclei are stained with DAPI (blue).

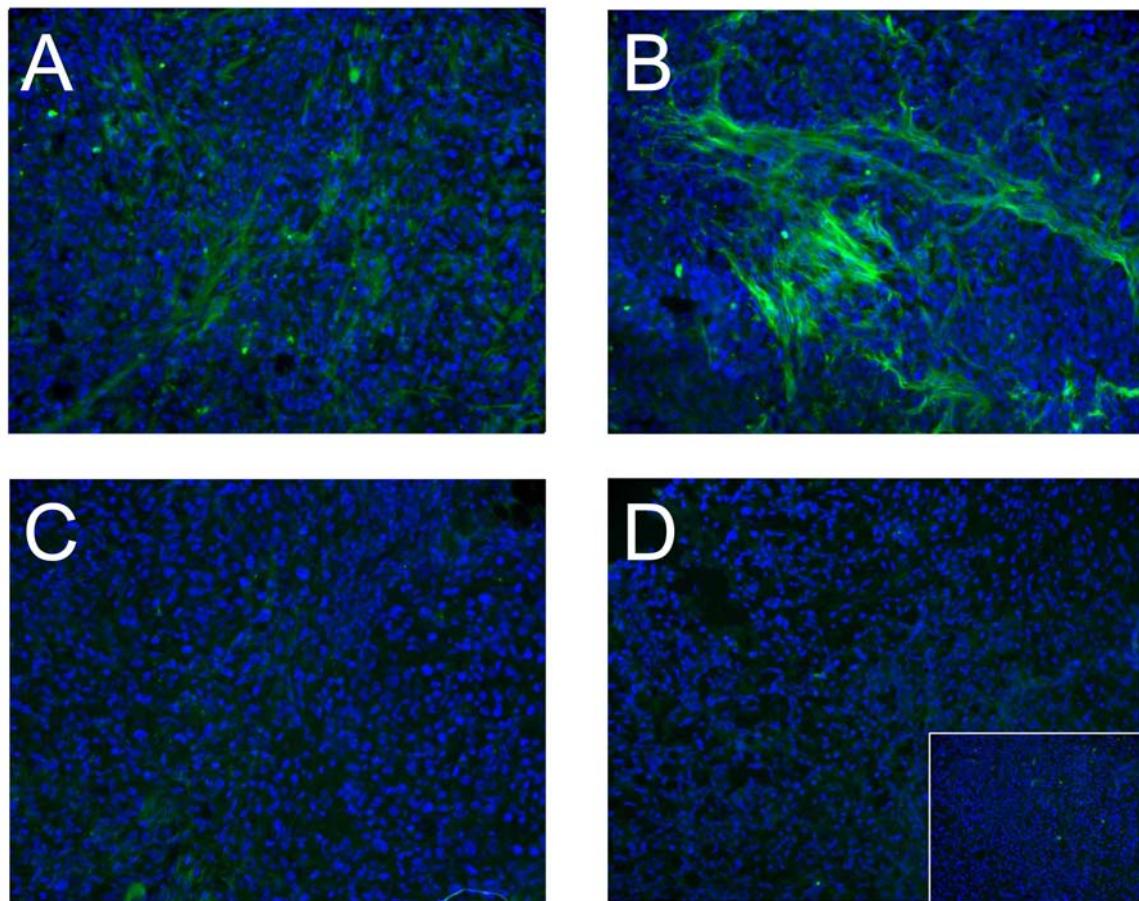


Figure 2 Mice with DU145 prostate tumors were tail vein-injected with 500 µg of CF-conjugated CLT1 (A), CLT1 QA (B), CLT1 NA (C), CLT1 KA (D) or ACLT1 (D, inset). After 4 h, the mice were perfused with PBS. Histological analysis using fluorescence microscopy showed that CLT1 QA produced stronger fluorescence (green) in the tumor stroma than CLT1 (A-B). Injection of CLT1 NA, CLT1 KA or ACLT1 did not produce relevant fluorescence (C-D). Nuclei are stained with DAPI (blue).

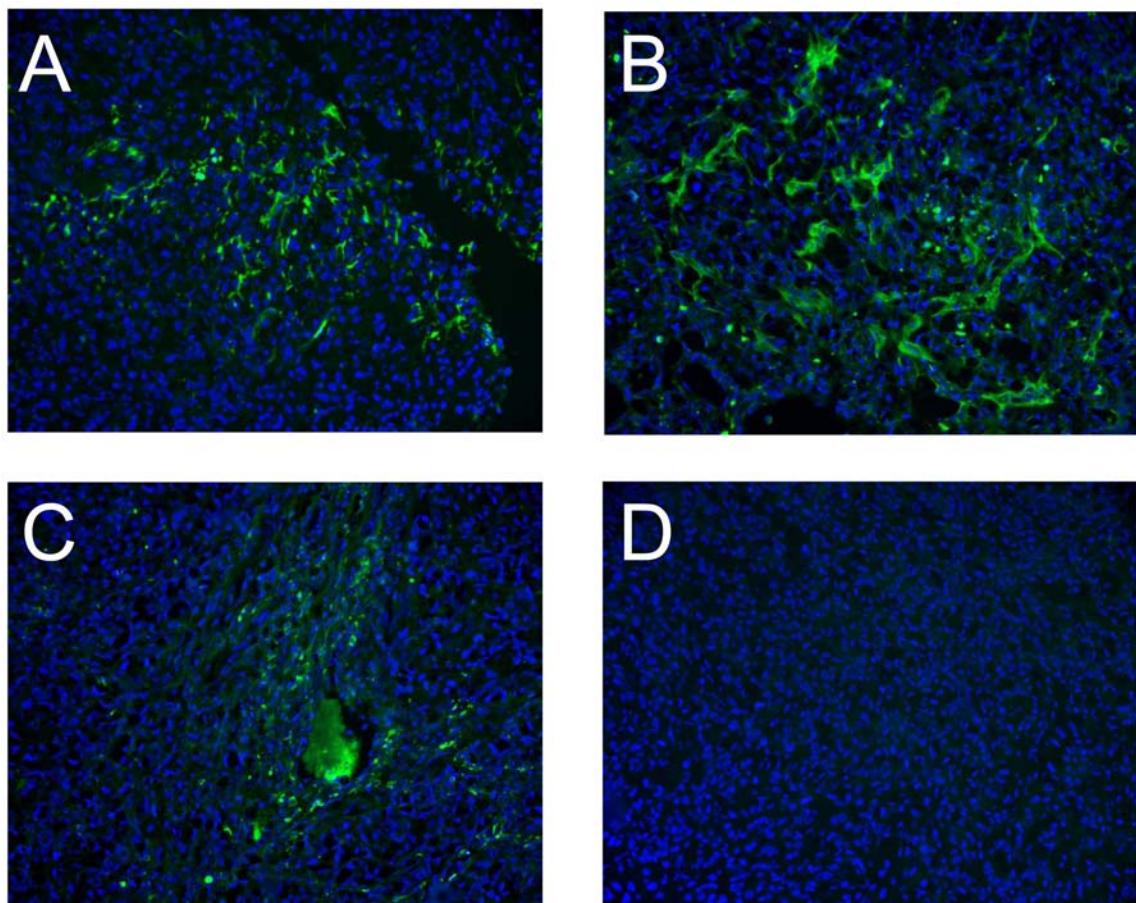


Figure 3 Mice with DU145 prostate tumors were tail vein-injected with 500 µg of CF-conjugated CLT2 (A), CLT2 SA (B), CLT2 NA (C) or ACLT2 (D). After 4 h, the mice were perfused with PBS. Peptides that bound to the tumor stroma produced a unique green fluorescence. Nuclei are stained with DAPI (blue).

Key Research Accomplishment

The AKN motif appears to be critical for CLT1 tumor homing.

Reportable Outcome

None

Conclusion

Our preliminary results support our hypothesis that XKN is part of the CLT1 tumor homing motif. Further studies are needed to confirm this result and to determine the CLT2 tumor binding function.

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Appendices

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